POPULATION GENETICS OF THE KOALA (*PHASCOLARCTOS CINEREUS*)

BY

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THESIS

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ABSTRACT

The koala (*Phascolarctos cinereus*) is an arboreal marsupial native to Australia. Koalas can be found in eastern Australia in the states of Queensland, New South Wales, Victoria and South Australia. The koala is listed as 'vulnerable' in Queensland and New South Wales, while in South Australia the koala's status is 'rare'. In the United States, the species is listed as 'threatened' under the U.S Endangered Species Act**.** In the early 1900s, koala fur was a valuable commodity, and the high demand for their pelts resulted in the slaughter of millions of koalas across Australia. In an effort to reintroduce koalas to their former range, Victoria was gradually restocked with animals from French Island, which were descended from a small number of individuals. Because the koalas from French Island descended from a small number of founders this resulted in a widespread translocation of koalas with low genetic diversity. Koalas are currently threatened by loss of habitat and urbanization due to increasing growth of the human population.

The goals of this study were: 1) to assess genetic diversity of the koala and 2) to determine the genetic structure of the koala. To achieve these goals, we sequenced 648 base pairs (bp) of the mitochondrial control region and developed 14 microsatellite markers. Queensland koala samples (N = 27) were collected from various United States zoos and are designated here as Queensland (zoo). Samples from wild koalas were collected from different localities in southern Australia, including: (BR) Brisbane Ranges (N = 23), (SR) Stony Rises (N = 24), (FI) French Island (N =1) and (KI) Kangaroo Island (N = 9). Mitochondrial control region haplotype diversity was extremely low in koalas from southern Australia. Koalas from BR, SR, FI and KI carried only haplotype (KCR1), compared to koalas from Queensland (zoo), which carried 8 haplotypes. We compared our 648 bp of koala mtDNA control region with previously published

koala haplotypes from Houlden et al., (1999), and found one novel haplotype (KCR10) in our Queensland (zoo) koalas, all other mtDNA sequences matched haplotypes described by Houlden et al., (1999).

Genotyping of 14 microsatellite markers showed low allelic diversity in koalas from BR and SR. Allelic diversity in koalas from BR and SR ranged from 2-5 alleles per locus. Three microsatellite markers (*Phci21*, *Phci23* and *Phci27*) were monomorphic in BR, while two were monomorphic (*Phci23* and *Phci27*) in SR. Allelic diversity for Queensland (zoo) koalas was much higher than for BR and SR koalas, with a range of 3-11 alleles per locus, and an average of 5 private alleles per locus. Observed heterozygosity ranged from a low Ho = 0.39 in koalas from BR to a high of Ho = 0.72 in the Queensland (zoo) koalas. One microsatellite marker (*Phci17*) deviated from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.0005$). Linkage disequilibrium was detected after Bonferroni correction ($P < 0.0005$) in one marker *Phci15.* Modified Garza-Williamson values were low, and ranged from 0.11 to 0.62 in the BR and SR koalas, which suggested past population size reductions and loss of alleles after a bottleneck or founder effect.

Population pairwise F_{ST} estimates showed significant genetic differentiation between the Queensland (zoo) koalas and the BR ($F_{ST} = 0.32$) and SR koalas ($F_{ST} = 0.29$). A low F_{ST} value was estimated between BR and SR koalas ($F_{ST} = 0.02$). Bayesian clustering analysis performed using STRUCTURE suggested the presence of two genetically distinct clusters: 1) Queensland (zoo) cluster, and 2) BR and SR cluster. *Ad hoc* methods to determine the number of partitions provided support for K = 2 for all models. Multivariate principal coordinate analysis (PCoA) implemented in GenAlEx was used to demonstrate genetic distinctiveness between koala populations. The PCoA determined two major groups based on the patterns found within a

distance matrix generated by pairwise individual-by-individual comparisons. The first group was defined by koalas from Brisbane Ranges and Stony Rises, which overlapped on the first principal component. The second group consisted of koalas from Queensland (zoo), which were distinct from the southern koala group. The major patterns found by PCoA mirror the same clustering pattern as the one suggested in the STRUCTURE analysis. The PCoA analysis demonstrated the distinctiveness between Queensland (zoo) koalas and southern koalas.

Our results showed that genetic diversity in koalas from southern Australia is low compared to koalas from Queensland (zoo). Limited haplotype and allelic diversity were revealed in koalas from BR, SR, FI and KI. Koalas in southern Australia have experienced severe population decline, which may have caused the loss of alleles from their gene pool. The translocation of koalas into BR and SR did not add any substantial genetic variation to the populations. The findings of this study may have implications for koala management in Australia and zoos in the US.

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TABLE OF CONTENTS

CHAPTER 1: Introduction and Review of the Literature.....……...1

Biology and evolution of the koala ……...……..1

Disease and threats ……..4

European settlement and the koala fur trade ……...5

Current conservation status ……...……...…....6

Genetic markers for studies of population structure ……...6

Population structure of the koala……8

CHAPTER 2: Mitochondrial genetic patterns among koalas (*Phascolarctos cinereus*).....11

Introduction……11

Materials and methods…….....12

Results…….…….....14

Discussion…….....…….....16

Conclusion…….....…….....19

Tables and Figures…….…….....21

CHAPTER 3: Examining genetic diversity and determining genetic structure in koala populations..... 31

Introduction…….....31

Materials and methods……...32

Results…….....36

Discussion……..39

Comparison of mtDNA haplotypes and microsatellites DNA patterns…….....42

Conclusion…….43

Tables and Figures ……45

Literature Cited……...54

CHAPTER I

Introduction and Review of the Literature

Biology and evolution of the koala

The modern koala is the only living member of the family Phascolarctidae (Dickman 2007; Jackson 2007). The modern koala family Phascolarctidae, appears to have evolved from a group of marsupials known as the diprotodonts (Black et al., ,2014; Martin et al., , 1999). The closest relative to koalas are the wombats of the family Vombatidae (Jackson 2007). In the phylogenetic tree of marsupials, koalas and wombats form a monophyletic clade (Black et al., , 2014). The two families diverged in the late Eocene, around 35 to 40 million years ago (mya) (Black et al., 2014).

A warming trend, beginning in the late Oligocene and peaking in the mid-Miocene favored the formation of vast lakes and rivers and the growth of rainforests (Black et al., 2014; Dickman 2007). The peak of marsupial diversity in Australia is linked to warm, wet climates during this early-middle Miocene epoch (Black et al., 2014; Dickman 2007). The fossil record indicates that a variety of animals, including koalas, kangaroos, possums, marsupial lions, and smaller carnivores were living in the rainforests (Dickman 2007). The early Miocene rainforest assemblages sustained sympatric species of koalas, with as many as three species *Priscakoala lucyturnbullae*, *Nimiokoala greystanesi*, and *Litokoala dicksmithi* recorded from a single fossil site (Black et al., 2014). A variety of vegetation present during this epoch, including *Eucalyptus,* sustained greater diversity of koalas than at any other period in geological history (Black et al., 2014; Dickman 2007). Climatic conditions again began changing 15 mya during the mid-Miocene, and Australia went from wet climate and rainforest to cool, dry conditions (Dickman

2007; Jackson 2007). This meant that rainforests were replaced by grasslands and by plants that could tolerate the arid conditions (e.g., *Eucalyptus*) (Dickman 2007; Jackson 2007).

By the late Miocene (10.4-5.2 Ma) the arid conditions of the continent caused significant extinctions of entire mammalian families that were unable to adapt to the changes (Dickman 2007). A similar trend is evident within the koala family Phascolarctidae with the extinction of some rainforest-adapted koala lineages (*Nimiokoala, Litokoala, Priscakoala*) (Black et al., 2014). Nine genera and at least 16 species of koala are known, including: *Invictokoala* (Pleistocene), *Phascolarctos* (Pleistocene – Pliocene), *Koobor* (Pliocene), *Encore LF* (late Miocene), *Litokoala* (middle Miocene – late Oligocene), *Nimiokoala* (early Miocene – late Oligocene, *Priscakoala* (early Miocene), *Perikoala* (late Oligocene), and *Madakoala* (late Oligocene). The spread of Australia's grasslands gave way to the emergence of the first megafauna 1.75 Ma. Among these were giant kangaroos, wombats the size of rhinoceros, and marsupial lions (Dickman 2007). Much of the megafauna persisted until the arrival of humans in Australia some 45,000- 50,000 years ago but then disappeared (Dickman 2007). By the last glacial maximum (18,000- 20,000 years), much of the megafauna had disappeared, but several other large species, including the modern koala had survived (Dickman 2007; Jackson 2007).

The koala is an arboreal mammal that can be easily recognized; they are short, have large round ears, thick fur, and a dark leathery muzzle (Jackson 2007; Martin et al., 1999). Their size and color, however, vary based on their geographic range (Jackson 2007; Martin et al., 1999). A Queensland koala has light grey fur and weighs around 6 to 8 kg, while the Victorian koala has a brown fur and weighs between 9-12 kg. The male koala tends to be larger than the female koala. (Jackson 2007; Lee and Martin 1988; Martin et al., 1999). The koala has been classified into three subspecies each corresponding to their range within an Australian state: *P.c. adustus* in

Queensland, *P.c. cinereus* in New South Wales and *P.c. victor* in Victoria (Lee and Martin 1988; Martin et al., 1999). Given that the koalas' natural range is continuous and shows substantial variation across state borders this variation may be clinal rather than discrete (Lee and Martin 1988; Martin et al., 1999).

Koalas feed primarily upon the leaves of *Eucalyptus*, although a variety of other non-eucalyptus trees are used by koalas for food and shelter (Jackson 2007; Martin et al., 1999). The low nutritional value and high toxicity of eucalyptus leaves renders them not only unpalatable but poisonous to other mammals (Jackson 2007). However, the koala has evolved to tolerate and digest the tree's toxic leaves. The slow metabolism of koalas allows the animals to survive on a very low intake of protein and energy (Jackson 2007).

Breeding season for koalas typically lasts from October to May (Lee and Martin 1988; Martin et al., 1999). During the breeding season, adult males move more frequently, over greater distances, and have larger home ranges (Jackson 2007). In a socially stable breeding environment, koalas coexist in overlapping home range areas (Jackson 2007). Within each animal's home range there are a small number of trees that are visited repeatedly, some of which are shared with other individuals in the population (Jackson 2007; Martin et al., 1999). Male koalas tend to have a larger home range than females. Males are polygynous, their home range tends to overlap with those of several adult females (Jackson 2007). While the movements of koalas in a socially stable breeding society are sedentary and localized, koalas that have left their maternal home range and are dispersing are capable of moving long distance (Jackson 2007; Martin et al., 1999). Koala dispersal movements have been recorded generally to be in the order of 1-3 km, although can occasionally be as high as10 km (Dique et al., 2003).

Disease and threats

Since European settlement, millions of hectares of forest and eucalyptus habitats have been cleared, mainly in Queensland and New South Wales (Jackson 2007). Today, ongoing habitat loss, urbanization, dog attacks and car accidents are threatening the koala (Phillips 2000). In addition to habitat loss, another threat to the koala has been diseases. Koalas are infected with *Chlamydia*, a gram-negative bacteria that has detrimental effects on its host (Brown et al., 1987). There are two chlamydial species that currently infect the koala, *C. pecorum* and *C. pneumoniae* (Jackson et al., 1999). A range of clinical conditions and syndromes caused by chlamydial infection have been observed, such as: kerato-conjunctivitis, rhinitis/pneumonia complex, urinary track disease, and reproductive track disease. Koala populations in Queensland and northern New South Wales have higher prevalence of chlamydial infection (Polkinghorne et al., 2013). Meanwhile, isolated populations in southern Australia (e.g., Magnetic Island, French Island and Kangaroo Island) are shown to be free of *Chlamydia* (Polkinghorne et al., 2013). It is thought that each of these populations was established using *Chlamydia*-free koalas (Lee and Martin 1988; Martin et al., 1999).

Leukemia and lymphoma also threaten the koala and they are linked to a retrovirus that is changing the koala genome. The koala retrovirus KoRV is an exogenous (horizontally transmitted) retrovirus that was discovered to be in the midst of becoming an endogenous (vertically transmitted) part of the koala (Stoye 2006; Tarlinton et al., 2006). To date, KoRV is the only known retrovirus that is currently in the midst of invading a host germ line. All northern koala populations are KoRV provirus-positive compared with southern populations that have a lower prevalence of KoRV (Simmons et al., 2012).

European settlement and the koala fur trade

Before the arrival of the European, koalas were hunted for food and clothing by the local indigenous Australians, which kept the koala population low (Jackson 2007; Lee and Martin 1988). Due to this hunting, at the time of European settlement in 1788 koalas were rarely seen. As European settlement decimated the indigenous human population in eastern Australia, koala numbers increased dramatically and by the 1890s the species was quite common. By the early 20th century, koalas had gained popularity among consumers for their fur and were heavily hunted for their pelts (Martin et al., 1999). The koala's fur is thick, warm and durable and was considered a valuable commodity in the international fur trade. The high demand for marsupial pelts for the fur trade led to the death of millions of koalas. One million koala skins were sold in the open season of 1919 and as many as two million were estimated to have been sold in 1924 (Martin et al., 1999). By the beginning of the 1930s koalas had been decimated and the species was at the brink of extinction. The koala fur trade resulted in a rapid population decline and localized extinctions across Australia. Koala populations were nearly extinct in Victoria and completely extirpated in the south-east of South Australia (Jackson 2007). Thus, European settlement in Australia, combined with the koala fur trade of the 19th century, caused tremendous declines in koala populations and many of them have been unable to recover.

In the early 1920s, towards the end of the fur trade, a translocation program run by the Victoria government was created to 1) reintroduce koalas to their former range from which they were thought to have disappeared since European settlement and 2) to reduce koala abundance at overpopulated sites. Thousands of koalas were translocated from French Island and Phillip Island stock populations to Victoria, Kangaroo Island and South Australia. The koala population in French Island, off the coast of Victoria, had been founded using as few as four koalas (Jackson

2007). As a result, the koalas from French Island are highly inbred with low levels of genetic diversity. Because the koalas from French Island lacked genetic diversity, this resulted in a widespread translocation of koalas with low genetic diversity (Houlden et al., 1996b).

Some areas of south-eastern Queensland and New South Wales have experienced, over the last few decades, high rates of growth in their human populations. Such growth has been detrimental for koalas as their habitat continues to be lost to both urbanization and tourist development. Urbanization is accompanied by habitat loss and increase in koala mortality rates. Habitat loss is the most serious conservation issue for koalas in New South Wales and south-eastern Queensland.

Current conservation status

The legal status of the koala in Australia varies by state or territory. In Queensland, the koala is listed as 'vulnerable' under the *Nature Conservation Act of 1992*; in New South Wales it is listed as 'vulnerable' under the *Threatened Species Conservation Act of 1995*; in Victoria the koala status is protected wildlife under the *Wildlife Act of 1975*; while in South Australia the koala's is protected under the *National Parks and Wildlife Act of 1972* (Jackson 2007). The U.S. Fish and Wildlife Service listed the species as 'threatened' under the *Endangered Species Act of 1973.* The koala's listing means that United States federal agencies must consider the impact of their actions on the koala, and prohibits any commercial activity or trade in koalas within the United States of America, except under a 'threatened species' permit (Hilton-Taylor et al., 2000).

Genetic markers for studies of population structure

<u>Mitochondrial DNA</u>

The unique characteristics of mitochondrial DNA make it a useful marker for studies of population and evolutionary genetics. Mitochondrial DNA or mtDNA, is found in the cells of Eukaryotes. Each cell contains several hundred copies of the mitochondria, each of which may have several copies of its own genome (Dawid and Rastl 1979). The high copy numbers allow for easy PCR amplification of mtDNA. Unlike nuclear DNA, mtDNA is inherited through the maternal lineage only. The evolutionary rate of mtDNA is higher than that of nuclear DNA leading to different levels of variability (Moritz et al., 1987). The mitochondrial control region is a non-coding region whose main function is to regulate replication and transcription (Gemmell et al., 1996).

Nuclear microsatellite markers

Microsatellites are short tandem repeats (STRs) of DNA sequence. Ever since the 1980s, microsatellite loci have been widely used in genetic studies, and have proven to be highly polymorphic (Ellegren 2004). Their ubiquitous distribution in the genomes of Eukaryotes make them very valuable markers (Ellegren 2004). In addition, microsatellites are valuable because primers developed in one species may be used in related taxa; however mutations in primer annealing sites may cause the occurrence of null alleles (Pemberton et al., 1995). Microsatellites have been widely used in studies of genetic diseases, population biology, and genetic structure. These markers tend to be under neutral selection with high mutation rates, but some loci are found in exons, and these may be under selective pressure. Microsatellites have different repeat sizes (di-, tri-, tetra- penta- and hexa- nucleotides), and are more desirable than other similar markers (e.g., minisatellites) because they are highly polymorphic, have smaller allele sizes (<1kb), have a narrower size range, and can be easily amplified by polymerase chain reaction (PCR) (Bruford 1993). Unique flanking regions allow each microsatellite to be analyzed as a

single locus, which can be amplified by PCR and scored for length polymorphism after electrophoresis (Guichoux et al., 2011). The flanking DNA regions of microsatellites are highly conserved within a species and thus can be used for creating unique PCR primers for each locus. Attaching a fluorescent tag (e.g., M13 primer) to the microsatellite primers will yield fluorescent allele products that can vary in size, and can be combined with other loci marked with different colors on the same gel to save time and resources (Boutin-Ganache et al., 2001). The mutation process in microsatellites sometimes occurs through polymerase slippage at DNA replication in a stepwise fashion, which decreases or increases the number of repeats by one unit (e.g., allele sizes at a dinucleotide locus differ by an even number of repeats) (Ellegren 2004).

Population structure of the koala

The first microsatellites markers for koalas were isolated by Houlden et al., (1996a) using genomic DNA from a Kangaroo Island sample. The characterization of six microsatellite loci revealed differences in allelic diversity (A) and expected heterozygosity (He) between four northeastern and six southeastern Australian populations. Koalas from the northeastern region had significantly higher levels of allelic diversity (A = 11.5±1.4) compared to the southeastern populations (A = 5.3±1.0). Heterozygosity levels were also higher in the northeastern populations (He = 0.851) compared to southeastern (He = 0.436). Cristescu et al., (2009) developed 11 microsatellite markers using Kangaroo and French Island koalas. Using the new 11 microsatellites and four additional loci from Houlden et al., (1996a), Cristescu and colleagues found an average 3.8 alleles per locus in French Island and an average 2.4 alleles per locus in Kangaroo Island (Cristescu et al., 2009).

Other studies have incorporated analyses of mtDNA to study the genetic structure of koala populations in Australia. A study by Taylor et al., (1997) using restriction enzymes in five

southern koala populations found only two mitochondrial haplotypes. Sequence divergence between haplotypes 1 and 2 was 0.12% and the within-population nucleotide diversity (π) ranged from zero (Kangaroo Island) to 0.03% (French Island). The study concluded that low mtDNA diversity in southern koalas is consistent with the observation that the koala translocation program has homogenized the gene pool in those populations involved in the program. Houlden et al., (1999) surveyed 16 populations distributed along the eastern coast of Australia, sequenced 860 bp of mitochondrial control region and revealed 18 haplotypes. Some of the haplotypes were shared between populations (e.g., H3, H5. H7, H10 and H18), with H18 being the most common haplotype in Victoria and South Australia. Other haplotypes were restricted geographically and were unique to a given population. Average nucleotide diversity within-population ($\pi = 0.16 \pm 0.001$) and haplotype diversity ($H = 0.180$) was low, while pairwise comparison between populations revealed an average nucleotide divergence of 0.51% (Houlden et al., 1999). Fowler et al., (2000) studied genetic diversity and patterns of gene flow in five southeastern Queensland koala populations. A 670 bp fragment of the control region was sequenced and four additional haplotypes were revealed, in addition to the 18 haplotypes previously found by Houlden et al., (1999) (Fowler et al., 2000). The number of control region haplotypes in a single population ranged from one to five, with an average within-population haplotype diversity of 0.379 ± 0.016, and nucleotide diversity of 0.22%. Nucleotide divergence between populations averaged 0.09% and ranged from 0.00 to 0.14%. This study suggested that female dispersal is limited in the southeastern region of Queensland and that further analysis using nuclear genetic diversity may reveal whether this is a general characteristic of koalas (Fowler et al., 2000). Furthermore, Lee et al., (2010) incorporated both microsatellite markers and mtDNA to determine the genetic structure of 512 koalas from ten Southeast Queensland locations and one island. This

comprehensive study detected 16 haplotypes, of which eight were new haplotypes, using 626 bp sequence of the control region. Haplotypes were geographically distributed by inland or coastal regions. The inland region had five different haplotypes (Haplotypes D, H, K, I and O), while the coastal region had ten haplotypes that were shared among the regions, suggesting historical gene flow. Bayesian clustering analysis using six microsatellite from Houlden et al., (1996) showed grouping of koalas from the coastal region into one cluster and second cluster with inland regions. Moreover, a study using ancient koala DNA showed that haplotypes were identical to those found in modern koala populations, suggesting that low mtDNA diversity has been present prior to the population decline caused by hunting for the fur trade (Tsangaras et al., 2012). In addition, this study also suggests that the arrival of the natives to Australia and climate changes in the late Pleistocene may have been the early events that caused the reduction of the koala's genetic diversity (Tsangaras et al., 2012).

CHAPTER 2

Mitochondrial genetic patterns among koalas (*Phascolarctos cinereus*)

Introduction

The koala is a threatened, arboreal marsupial that is native to Australia (Martin et al., 1999). Koalas in Australia have experienced population decline, extinction and over hunting throughout their history. During the 1800s, koala populations were found across the southern state of Victoria, but many of these populations were decimated after millions were hunted for their fur (Lee and Martin 1988). While koalas became nearly extinct in the southern states of Australia, their populations in the north were fragmented and experienced periodic population crashes (Jackson 2007; Martin et al., 1999). Although koalas were once widespread across the eastern coast of Australia, today their populations are patchily distributed and isolated from each other by areas of unsuitable habitat or cleared land (Jackson 2007; Martin et al., 1999). Currently, koala populations are commonly found-across the eastern coast of Queensland, New South Wales, Victoria and South Australia (Jackson 2007; Martin et al., 1999).

The mitochondrial control region is non-coding and is the most polymorphic region of the mitochondrial genome. The high rates of evolution of the mammalian mitochondrial control region allow for this marker to be reliable for studies of population genetics and for assessing genetic variation in natural populations. Therefore, it is commonly used as a genetic marker for assessing population structure. In this study we examined genetic diversity using mitochondrial control region sequences in geographically distant koala populations in Queensland (zoo), Victoria and Kangaroo Island. Our objective was to measure genetic diversity using koala

mitochondrial control region sequences from 4 locations in southern Australia and from Queensland (zoo) individuals.

Materials and methods

We used koala genomic DNA from zoo koalas from northern Australia designated here as "Queensland (zoo)" and from southern Australia, including Victoria (Stony Rises, Brisbane Ranges, French Island) and South Australia (Kangaroo Island). The koala samples collected in the zoos are from the *P.c. adustus* subspecies, (which can be found in Queensland), Australia, while the southern koalas are from the *P.c. victor* subspecies (which can be found in Victoria). Whole blood samples from Queensland (zoo) were collected during routine veterinary care from various US zoos, including: Cleveland Zoo, Colombia Zoo, Dallas Zoo, San Diego Zoo and San Francisco Zoo. The koala samples from Victoria and Kangaroo Island Australia were collected for use in a previous study (Taylor et al., 1997). The sample size for each of the populations was: Queensland (n=26), Stony Rises (n= 24), Brisbane Ranges (n= 23), Kangaroo Island (n= 9) and French Island (n= 1).

A 648 base pair (bp) segment of the mitochondrial control region was bidirectionally sequenced for 83 individuals. Polymerase chain reaction (PCR) amplification was carried out using the following forward and reverse primers: PCI_CR_1F (AAATAACAACCAACACTCACATCC 3') and PCI_CR_NR (TTCTAGGTACGTCCGCAATCT 3'). The total PCR reaction volume was 25 µl, consisting of 0.5 µl of DNA template, 20 µM final concentration of each oligonucleotide primer, 25 mM $MgCl_2$, 10 mM of each of the dNTPs (Applied Biosystems Inc. [ABI], Foster City, CA), and 10X PCR Buffer II (ABI) with 0.04 units/µL final concentration of AmpliTaq Gold DNA

Polymerase (ABI). PCR conditions consisted of an initial 95°C for 9:45 min; with cycles of 20 s denaturing at 94°C, followed by 30 s annealing at 60°C (three cycles); 58, 56, 54, 52°C (five cycles each temperature); or 50°C (last 22 cycles), followed by 1.5 min extension at 72°C, with a final extension after the last cycle of 7 min at 72°C. Prior to sequencing, primers and dNTPs were removed from the PCR products with Exonuclease I (USB Corporation, Santa Clara, CA) and shrimp alkaline phosphate (USB Corporation). Sequences in both directions were generated using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) with 2.5 µL of purified PCR products and 2 µM primer and resolved on an ABI 3730XL capillary sequencer. The software Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI) was used to edit chromatograms, assemble contigs for each amplicon, and concatenate the mtDNA sequences.

Previously published koala control region sequences were downloaded from the NCBI Nucleotide Database and aligned together with our control region sequences using MUSCLE implemented in MEGA6 (Tamura et al., 2011). The accession numbers from the previous studies are: KF745869-KF745875 (Seddon et al., 2013), AJ012057-AJ012064 (Fowler et al., 2000), GQ851933- GQ851940 (Lee et al., 2010), and AJ005846-AJ005863 (Houlden et al., 1999).

The control region sequence alignments were trimmed to 626 bp (Lee et al., 2010) or to 648 bp (this study) to account for differences in the length of previously published data sets. Two median-joining (MJ) networks (Bandelt et al., 1999) were constructed using the software NETWORK version 4.6. (http://www.fluxus-technology.com/sharenet.htm). The first haplotype network (A) was generated using our control region sequences with previously published mtDNA sequences and haplotypes frequencies from Houlden et al., (1999) for which haplotype frequencies by locality were available. For the second haplotype network (B), we combined our

control region sequences with mtDNA sequences from previous studies for which haplotype frequency was not always available (Fowler et al., 2000; Houlden et al., 1999; Lee et al., 2010).

Haplotype diversity and nucleotide diversity was calculated for the dataset that contained haplotype frequency information using Arlequin version 3.5 (Excoffier and Lischer 2010). Haplotype diversity was calculated for koalas grouped into the following populations: Queensland (zoo), Queensland (wild), New South Wales and Victoria. To evaluate the extent of population subdivision we performed an analysis of molecular variance (AMOVA) and computed pairwise F_{ST} values in Arlequin 3.5.

Results

Our first dataset included 648 bp of mtDNA control region sequences from 83 koalas from Queensland (zoo), Victoria and South Australia, and previously published haplotypes from Houlden et al., (1999), for which haplotype frequencies by locality were available. The localities with haplotype frequencies are: Gold Coast (GC), Mutdapilly (MT) and Springsure (SP) in Queensland; Campbelltown (CB), Coonabaraban (WNSW), Iluka (IL), Maitland (MT), and Nowendoc (NC) in New South Wales; Brisbane Ranges (BR), French Island (FI), Phillip Island (PI), South Gippsland (SG), Stony Rises (SR), Strzelecki Ranges (SZ), and Tubbut (TB) in Victoria; and Kangaroo Island (KI) in South Australia. The second dataset included 626 bp of mtDNA control region sequences from 83 koala samples and previously published haplotypes from four studies (Seddon et al., 2013; Lee et al., 2010; Fowler et al., 2000; and Houlden et al., 1999).

Median-joining network A (646 bp), shows the relationships among 22 haplotypes (KCR1-9, KCR18-30) with KCR1 having the highest frequency (Figure 1). Median-joining (MJ)

network B (648 bp) included 35 mtDNA control region haplotypes (KCR1 to KCR35) (Figure 3). Both haplotype networks displayed a star-like topology (Figure 1 and Figure 3). This is generally interpreted as indicative of a population that has recently expanded in size from a small number of founders following a population bottleneck. Haplotype KCR10 was found only among five koalas from Queensland (zoo) (Figure 2).

Haplotype diversity for Queensland (wild), New South Wales and Victoria was H= 0.7355 ± 0.03, H= 0.8346 ± 0.02, and H= 0.0708 ± 0.03, respectively (Table 1). Haplotype and nucleotide diversity in Queensland (zoo) were H = 0.7538 and π = 0.007, respectively (Table 1 and Table 2). The AMOVA results suggest that the source of structuring is among populations within groups having 41.24% of the variation, whereas 31.30% of the variation was found within populations and 27.46% of the variation was found among groups (Table 3). Accordingly, the fixation index was F_{ST} = 0.68 $P < 0.0001$ after 1023 permutation. Pairwise F_{ST} comparison between the localities showed low genetic differentiation among the southern Australian localities, while the northern Australian localities and the Queensland (zoo) population show higher levels of differentiation from the southern populations.

Our koala samples from southern Australia (BR, SR, FI and KI) showed low mtDNA haplotype diversity, compared to koalas from northern Australia and Queensland (zoo). Only one haplotype was found in our koala samples from southern Australia (Table 1). Interestingly, the Queensland (zoo) population had 10 haplotypes, while Queensland (wild) and Victoria had five haplotypes each (Table 2). However, the sample size for Victoria was about three-fold larger than the Queensland sample size, and yet the number of haplotypes was low (Table 1).

We also found evidence of genetic structuring in the populations, which is likely a reflection of the unique haplotypes found in some of the sampled regions (Figure 2 and Figure 4).

Discussion

Our results are consistent with previous findings which reported that koalas have low levels of mtDNA diversity (Fowler et al., 2000; Houlden et al., 1999; Taylor et al., 1997). Sequencing of 648 bp of the koala mitochondrial control region revealed nine haplotypes (Figure 3). One haplotype was detected in our samples from Brisbane Ranges, Stony Rises, French Island and Kangaroo Island, while eight different haplotypes were detected in koalas from Queensland (zoo) (Table 2). Similar levels of haplotype diversity were shown between our Queensland (zoo) koalas and the Queensland (wild) koalas (Table 1), which may be an indication that genetic diversity is being conserved through proper management. The haplotype diversity found in our Queensland koala samples from US zoos should be a close representation of the genetic diversity of the source population. The aim of captive management is to capture the species genetic diversity through a group of founders and preserve it in captivity. The original founding individuals are assumed to be a representative sample of the source population and the goal is to preserve the genetic composition of the source population across time by preserving the genetic diversity of the founders. However, founder contributions in most captive populations are highly skewed, usually due to uneven breeding of a small proportion of the founders. Genetic diversity potentially contributed by the underrepresented founders is at high risk of being lost due to genetic drift (Ballou et al., 2010). For management programs to maintain genetic diversity in captive populations it is important to maximize the population's effective size by maximizing the number of different breeding individuals, equalize the sex ratio of

breeders and rotate breeding among many animals so that each breeding group or pair produces similar number of offspring (Ballou et al., 2010). Our Queensland (zoo) koalas showed higher levels of mitochondrial haplotype diversity than the Queensland (wild) koalas. This may be due to zoos having obtained individuals that originated from subpopulations with different haplotypes.

In population genetics, the Wahlund Effect refers to the reduction in heterozygosity due to subpopulation structure, even if the subpopulations themselves are in Hardy-Weinberg equilibrium. Suppose there are two subpopulations P1 and P2 that are separated by a geographic barrier so that no gene flow occurs. Subpopulation P1 has a frequency of 1.0 for the A_1 allele (all individuals are homozygous for A_1) and subpopulation P2 has a frequency of 1.0 for allele A_2 (all individuals are homozygous for A_2). If individuals from subpopulation P1 are combined with an equal number of individuals from subpopulation P2, with each population providing an equal number of males and females, after one generation the frequency of A_1A_2 heterozygotes will be 0.5 (or 50%), while the frequency of A_1 and A_2 homozygotes will be 0.25 and 0.25, respectively. Although the Wahlund is not directly applicable to mtDNA, for which each individual carries only a single haplotype, an analogous process appears to have occurred. The founder koalas in zoos likely come from many different localities in Queensland, and thus represent greater diversity than may be found in wild koalas from a more limited number of locations.

Low levels of haplotype diversity were expected for the southern koalas (Table 1), which for a period of time, were hunted to near extinction, and as a consequence their population was subject to severe decline and local extirpation. To rescue and conserve what was left of the koala population in Victoria, a number of koalas were translocated to Kangaroo Island and French Island (Jackson 2007). These insular populations were later used to restock the southern

Australian mainland (Jackson 2007). The restocking of the populations in Victoria brought lasting consequences to that population's genetic diversity.

The decrease in number of koalas would suggest that the species subsequently exhibited reduced genetic diversity due to population bottlenecks. Several molecular markers have been used to examine the genetic variability of the Australian koala and have shown that genetic diversity within and gene flow between koala populations is low (Taylor et al., 1991; Taylor et al., 1997; Timms et al., 1993). Houlden et al., (1999) examined 860 bp of mtDNA control region in 208 koalas from 16 populations across Australia. Koala mtDNA diversity within populations averaged H = 0.18.

Genetic differentiation within the koala populations of Queensland and New South Wales was found to be high, while koalas within the populations of Victoria were little differentiated (Houlden et al., 1999), most likely due to their being derived from inbred individuals translocated from the French and Phillip Islands. Fowler et al., (2000) examined 670 bp of mtDNA from 96 koalas from five southeastern Queensland populations. Haplotype diversity within populations was estimated as H = 0.37, higher than (Houlden et al., 1999). Tsangaras et al., (2012) sequenced 201 bp of the mtDNA control region of 14 ancient koalas from museum skins. Four haplotypes were detected and all of them were present in modern koalas (Tsangaras et al., 2012). In other words, genetic diversity was already low in the 19th century, which suggest that limited mtDNA variability was present in northern koalas prior to the recent historical population decline.

Extinctions in Australia, attributed to Aboriginal arrival, climate change, or their combination, resulted in the disappearance of the continent's megafauna, including other species of koala (Roberts et al., 2001). Prior to this extinction event the modern koala (*P. cinereus*) co-

existed with a larger, now extinct species of koala (*P. stirtoni*), and the modern koala had a wider distribution that extended to south-western Australia (Jackson 2007; Roberts et al., 2001). The reduction in taxonomic diversity of koalas and reduction of the geographic range of the living koala since the Pleistocene, might have impacted on the genetic diversity of *P. cinereus*, (Black et al., 2014; Jackson 2007). Koalas may have experienced population expansions and subsequent crashes many times through the Quaternary, depending on the availability of habitat and the intensity of hunting after human arrival. A severe decrease in population size and subsequent reduction in genetic diversity is associated with a variety of threats in affected populations. Some of these threats include: inbreeding and reduction in fitness, expression of recessive deleterious alleles, and reduced resistance to disease (O'Brien 1994). Coincidentally, koalas are infected with two lethal pathogens: *Chlamydia* and the koala retrovirus (KoRV). KoRV is a major pathogen in wild koalas that compromises the immune system of its host (Hanger et al., 2000; Tarlinton et al., 2006). KoRV is an endogenous retrovirus that is associated with cancer in wild and captive koala populations (Tarlinton et al., 2008).

Conclusion

In this study, we sequenced 646 bp of the koala mitochondrial control region and used previously published koala mtDNA control region sequences to evaluate haplotype diversity in koala populations from northern and southern Australia. Our results show that mtDNA haplotype diversity is extremely low in koalas from southern Australia compared to koalas from northern Australia. Our southern populations carried only one haplotype, while the Queensland (zoo) population carried eight haplotypes. Among the haplotypes identified in our koalas, KCR10 was detected only five Queensland (zoo) koalas. The koala's genetic diversity may have been affected by a combination of environmental changes during the Pleistocene and more recent

episodes of overhunting. The combined pressure on koalas has likely limited the level of genetic diversity.

Tables and Figures

Table 1. Estimates of haplotype mtDNA diversity within four koala populations: Queensland (zoo) abbreviated QLD (zoo), Queensland (wild) abbreviated QLD (wild), New South Wales (NSW) and Victoria (VIC).

Population	QLD (zoo)	QLD (wild)	NSW	VIC
No. of haplotypes	8	5	10	5
Sample size	26	49	77	139
Haplotype diversity (SD)	0.7538 (± 0.07)	0.7355 (± 0.03)	0.8346 (± 0.02)	0.0708 (±0.03)
Nucleotide diversity (SD)	0.007194 (± 0.004058)	0.007695 (±0.004225)	0.006177 (± 0.003459)	0.000351 (±0.000460)

Table 2. Estimate of nucleotide diversity (π) for each location. Estimates of π were calculated using 648 bp of mtDNA control region using Arlequin. The locations in Queensland are: Gold Coast (GC), Mutdapilly (MT), and Springsure (SP). Koalas from US zoos are abbreviated as QLD (zoo). The locations in New South Wales are: Campbelltown (CB), Maitland (ML), Nowendoc (NC), Coonabaraban (WNSW), Maitland (ML), and Iluka (IL) and the locations in Victoria are: Brisbane Ranges (BR), South Gippsland (SG), Stony Rises (SR), Strzelecki Ranges (SZ), Tubut (TB), French Island (FI), Phillips Island (PI), and Kangaroo Island (KI). Haplotypes are shown on a map in Figure 2.

	Queensland				New South Wales					Victoria							
Locality	GC	MT	SP	QLD (zoo)	CB	ML	NC	WN SW	IL	BR	SG	SR	SZ	TB	FI	PI	KI
Sample size	19	16	14	26	22	8	20	9	18	30	19	29	12	1	16	13	19
No. of haplotypes	1	3	1	8	4	2	1	2	2	1	3`	1	3	1	1	1	1
π	0.00	0.008	0.00	0.007	0.002	0.003	0.00	0.002	0.008	0.00	0.00	0.00	0.001	na*	0.00	0.00	0.00

*Not applicable (na), nucleotide diversity was not calculated for this locality due to a small sample size.

Table 3. Analysis of molecular variance (AMOVA) using pairwise differences among groups of koalas

Source of variation	Sum of squares	Variance component	Percentage of variation (%)
Among groups	168.710	1.07255	27.46
Among populations within groups	155.250	1.61063	41.24
Within populations	174.797	1.22236	31.30
Total	498.757	3.90554	

Figure 1. Median-joining network "A" of 648 bp mitochondrial control region haplotypes in koalas from Queensland (zoo), Queensland (wild), New South Wales and Victoria. Median-joining network A was generated using the dataset for which haplotype frequency information was known. Circle size is proportionate to number of koala samples. Haplogroups are color coded. Haplogroups are defined by a separation of at least seven mutational steps between each haplogroup. For example, there are seven mutational steps between KCR5 and KCR9. Nucleotide differences are indicated (if > 1) by hatch marks. Haplotype KCR1 was the most common haplotype found in koalas from Victoria and Kangaroo Island. This is important because it shows that the koalas in these southern population have limited genetic diversity, which likely due to population bottlenecks and founder effects.

Figure 1 (cont.)

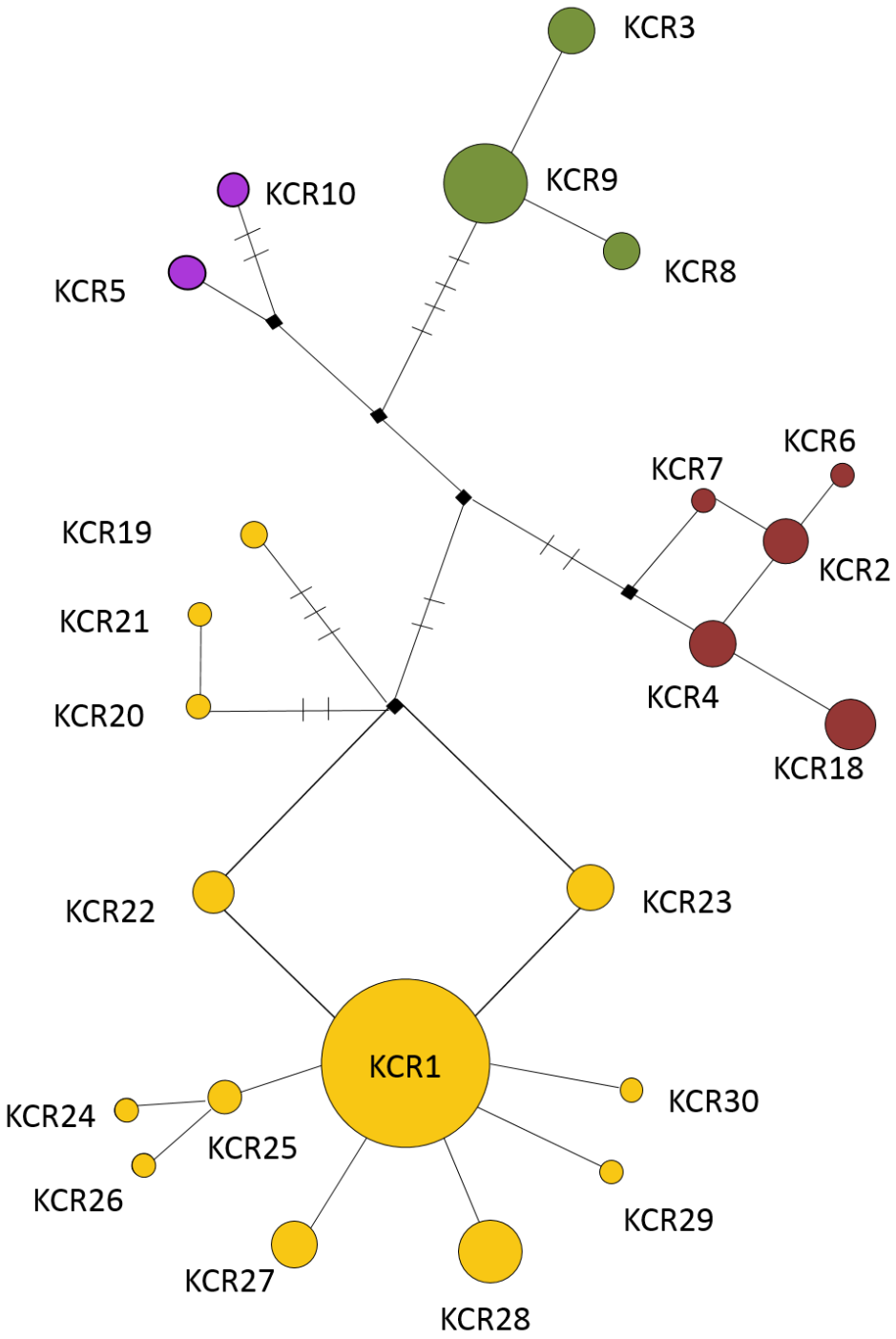


Figure 2. Distribution of koala 626 bp mtDNA control region haplotypes. The colors represent haplogroups (see Figure 1 for haplogroup designation). Pie charts are proportionate to haplotype frequency, while circles with only one haplotype are not proportionate to sample size. Zoo koalas are grouped in a separate pie chart and carry four haplogroups. One zoo koala (PCI-513017) carries haplotype KCR1. The locations from north to south are: Springsure (SP), Mutdapilly (MT), Gold Coast (GC), Iluka (IL), Nowendoc (NC), Coonabaraban (WNSW), Maitland (ML), Campbelltown (CB), Tubut (TB), Strzelecki Ranges (SZ), South Gippsland (SG), French Island (FI), Phillip Island (PI), Stony Rises (SR), Brisbane Ranges (BR) and Kangaroo Island (KI).

Figure 2 (cont.)

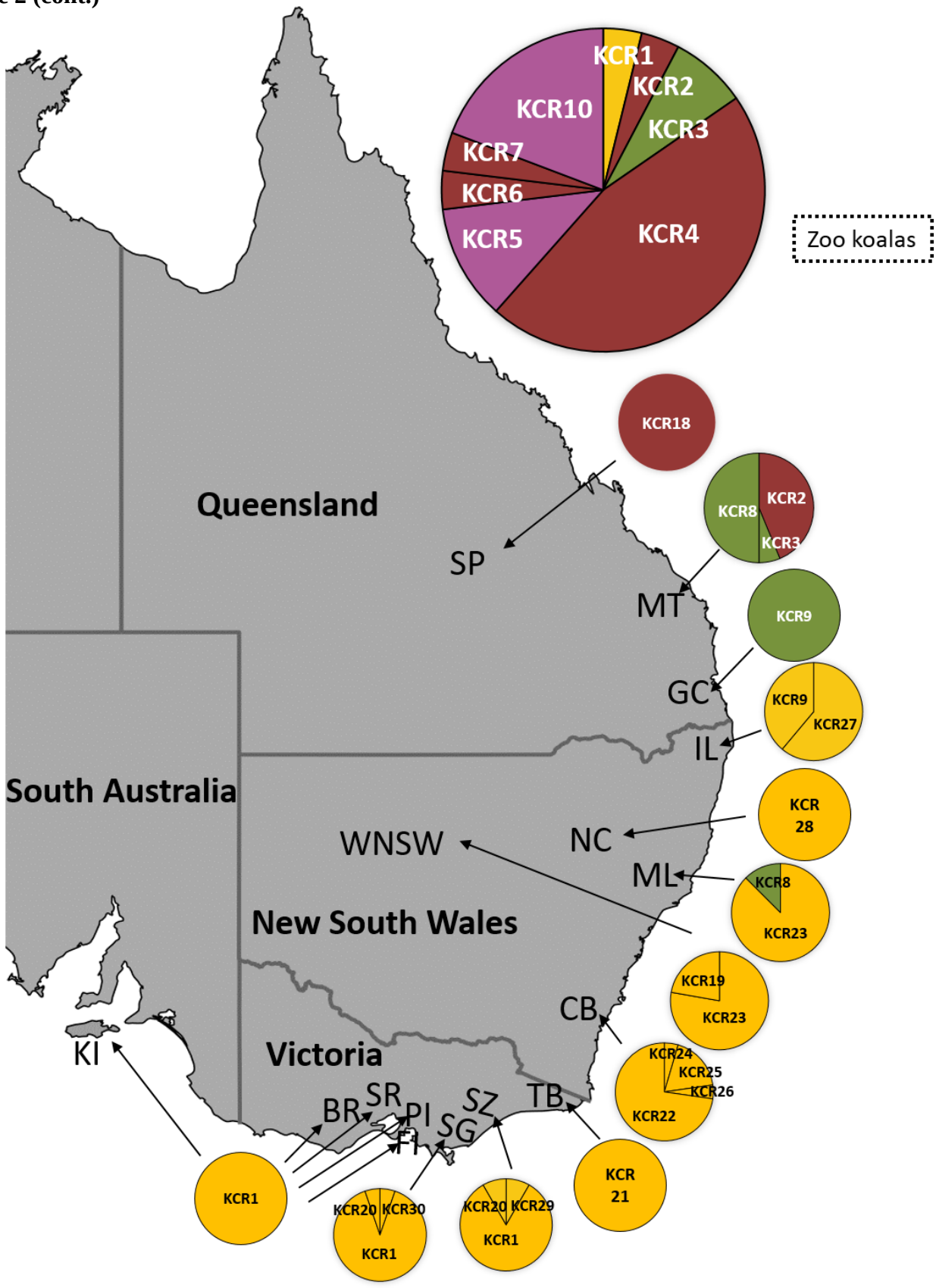


Figure 3. Median-joining network "B" using 626 bp of koala mitochondrial control region. This haplotype network was constructed using mtDNA sequences from previous studies, in which haplotype frequencies were not always reported (Seddon et al., 2013, Fowler et al., 2000, Lee et al., 2010, and Houlden et al., 1999), and 83 koala control region sequences from this study. Our koala sequences from Victoria and Kangaroo Island matched with a previously published haplotype (KCR1) from southern Australia (Houlden et al., 1999). The zoo koalas matched to haplotypes found in southeast Queensland (Seddon et al 2013, Lee et al., 2010 and Fowler et al., 2000). There was one haplotype KCR10 detected in zoo koalas only. The circles are not proportionate to sample size. The number of nucleotide differences is indicated (if>1) by hatch marks.

Figure 3 (cont.)

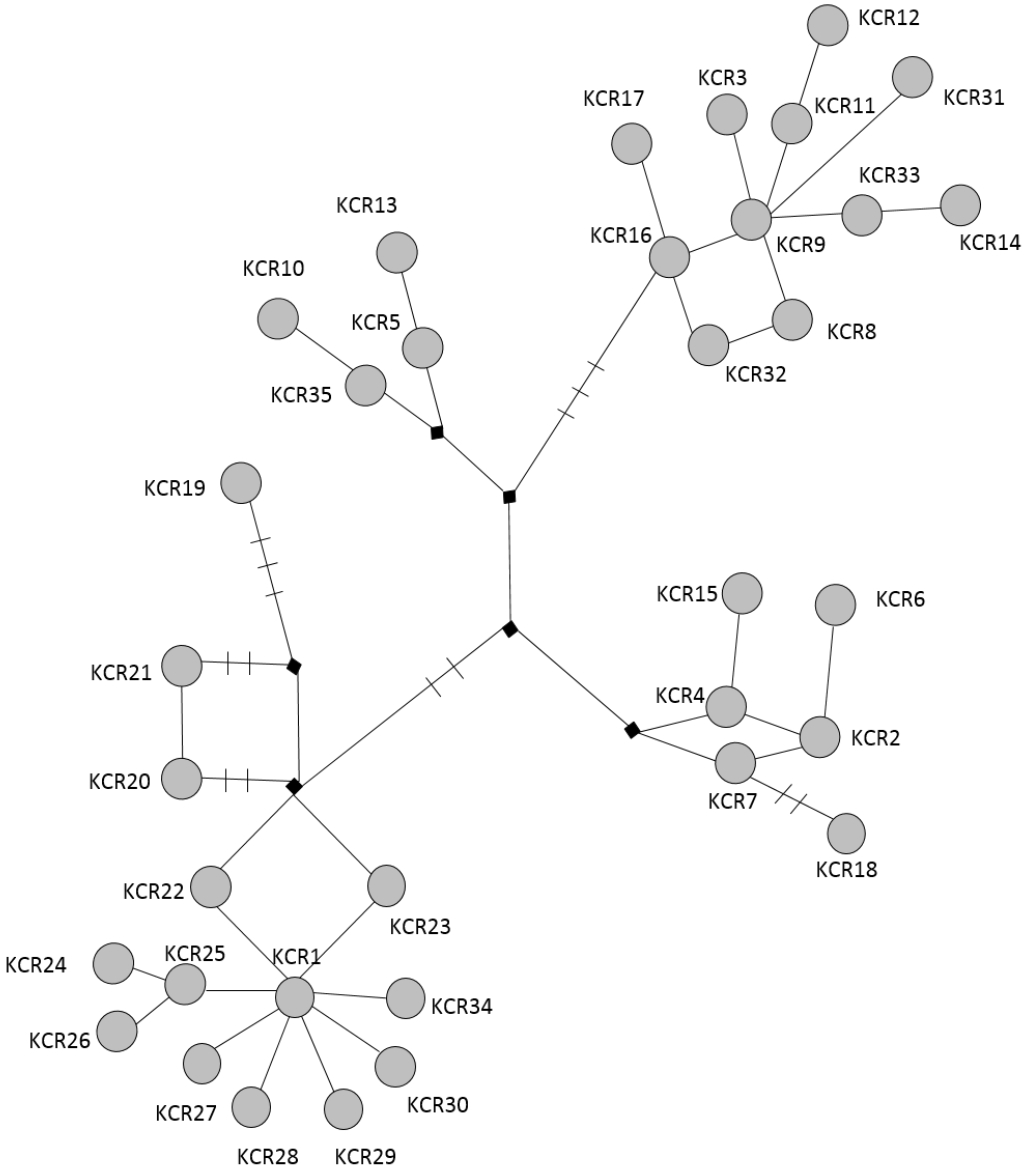
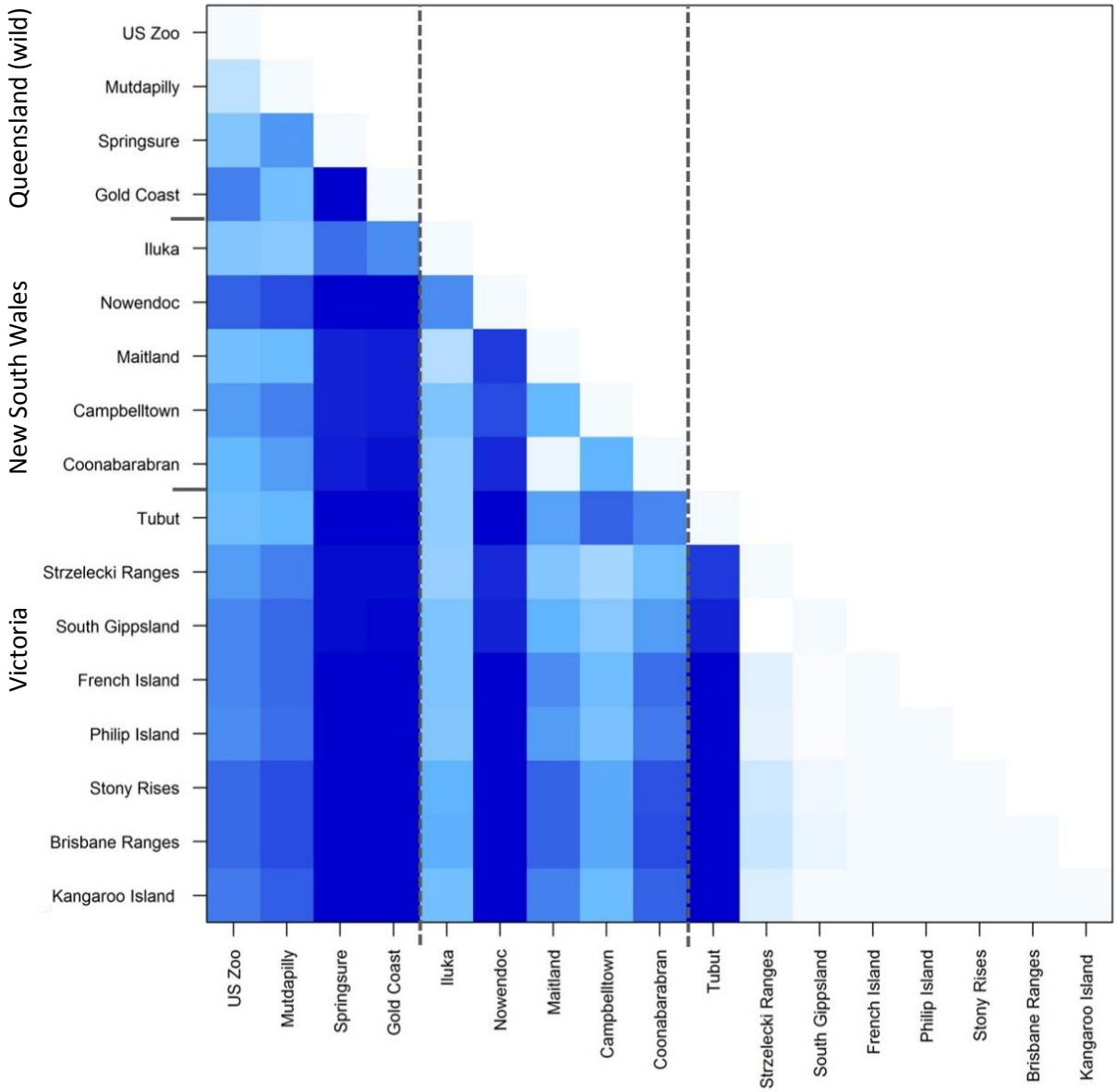


Figure 4. Pairwise F_{ST} matrix showing genetic differentiation between localities. Localities are in order from north to south. Color gradient from 0.0 (lightest blue) to 1.0 (darkest blue). Dark blue indicates high F_{ST}.



CHAPTER 3

Examining genetic diversity and determining genetic structure in koala populations.

Introduction

The koala (*Phascolarctos*) is listed as vulnerable by the Australian government, and as threatened under the U.S. Endangered Species Act. Populations of koalas in Victoria and South Australia went through severe population declines due to hunting in the 19th century, after European settlement. Koalas were reintroduced to their former range in Victoria and some parts of South Australia. Koalas from French Island, off the coast of Victoria, were used as the source population to translocate koalas into the Brisbane Ranges and Stony Rises (Martin et al., 1999). In Queensland, koalas are threatened by habitat loss and fragmentation, disease and predation (Department of the Environment 2013.). Currently, koalas that come from Queensland (nominally *P. c. adustus*) and are housed in US zoos are managed as a separate stock from southern Australian koalas, which include koalas from New South Wales (nominally *P. c. cinereus*) and Victoria (nominally *P. c. victor*). For convenience we will refer to the koala US zoo samples as Queensland (zoo).

In a previous study, we had we evaluated the genetic diversity of the Queensland (zoo) koalas using microsatellites markers. Fourteen microsatellite markers for Queensland (zoo) koala were initially developed and tested in ten unrelated individuals from the San Diego Zoo, USA (Ruiz-Rodriguez et al., 2013). DNA from one koala (Pci-SN404) was sequenced using 454 Next Generation Sequencing technology. A total of 35,942 sequence reads were obtained, with an average read length of 549 base pairs (bp). Microsatellites were identified, and primers were designed using MSATCOMMANDER 1.08. The number of alleles per locus ranged from 2 to 7,

with an average of 0.69 for observed and expected heterozygosity. Of the 14 markers, *Phci12*, showed evidence for deviation from Hardy-Weinberg equilibrium, while *Phci16* and *Phci19* had low allelic diversity (Ruiz-Rodriguez et al., 2014).

Our aim in this study was to examine and compare genetic diversity and structure in koalas from Queensland (zoo) and Victoria, Australia. Our main focus was to: 1) use microsatellite markers that were previously developed in the Queensland (zoo) koala and test them for effectiveness in two southern koala populations and 2) compare and contrast the genetic structure and diversity of two koala populations in Victoria with koalas from Queensland (zoo).

Materials and methods

For the study reported here, we used 10 polymorphic markers of the 14 that were previously published (Ruiz-Rodriguez et al., 2013), in addition to four newly developed markers. The other four previously developed microsatellite markers (*Phci12*, *Phci16*, *Phci28* and *Phci31*) were not included in this study for various reasons: *Phci12* was initially reported to be in deviation from HWE, *Phci16* had low heterozygosity, while *Phci28* and *Phci31* had too much missing data. These problematic markers were therefore not used.

Fourteen microsatellite makers were used in this study to genotype 73 koalas, including the 10 koalas from the San Diego Zoo. Four novel microsatellite markers were designed and are reported in this study for the first time. The development of the new markers followed the same procedure reported by Ruiz-Rodriguez et al., (2014).

Queensland (zoo) koala samples were collected by various zoos in the United States including: Cleveland Zoo, Columbus Zoo, Dallas Zoo, San Diego Zoo, and San Francisco Zoo. Blood samples from the Queensland (zoo) koalas were collected by veterinarians during routine

veterinary care and shipped to us in leak-proof containers. DNA was extracted from blood samples using a DNeasy Blood and Tissue Kit (QIAGEN), following the recommended protocol. Wild koala samples from Victoria, Australia, had been collected for use in a previous study (Taylor et al., 1997). A total of 27 samples of Queensland (zoo) koalas and 46 samples from Victoria were used in this study. The samples from Victoria (southern Australia) consisted of 22 samples from Brisbane Ranges (BR) and 24 samples from Stony Rises (SR). Koala samples from Kangaroo Island (n = 11) and French Island (n = 1) were also tested using the microsatellite markers, but these failed to amplify and were not considered further.

The PCR setup and algorithm were the same as used successfully by Ishida et al., (2012). Details of the PCR setup and thermocycling are found in the supplementary material of that publication (Ishida et al., 2012). All forward primers had attached a tail consisting of M13 forward sequence (5' TGT AAA ACG ACG GCC AGT), to enable labeling with a tag (Boutin-Ganache et al., 2001). Primer pairs were initially tested by PCR performed in a 15 µl reaction mixture that consisted of a final concentration of 200 µM of each dNTP, 1x PCR buffer II, 2 mM $MgCl_2$, 0.04 units/µl of AmpliTaq Gold Polymerase along with 1.2 µL of primer mix (primer mix recipe is listed below) and 0.5 µl of template DNA. For DNA that was of poor quality, 0.8 µg/µL final concentration of BSA (New England BioLabs Inc, Ipswich, MA) was included. Touchdown PCR was used with the following algorithm: initial 95°C for 10 min; with cycles of 15 sec at 95°C; followed by 30 sec at 60°C, 58°C, 56°C, 54°C, 52°C (2 cycles each), or 50°C (last 30 cycles); and 45 sec at 72°C; with a final extension of 30 min at 72°C (see below). An aliquot of each PCR product was examined on a 1.5 to 2% agarose gel with ethidium bromide. Amplicons were then diluted depending on the intensity of the image in the gel (a 5X dilution for dimmer bands and a 10X dilution for brighter bands) and electrophoresed on an ABI 3730XL

capillary sequencer at the UIUC Core Sequencing Facility. Microsatellite fragments were scored and binned with Genemapper Version 3.7 software (Applied Biosystems)

Fourteen primer pairs were used for genotyping to determine marker variability in 73 koala samples. Allelic diversity, observed heterozygosity and expected heterozygosity were calculated using ARLEQUIN (Excoffier and Lischer 2010) and GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were calculated using ARLEQUIN and GENEPOP, v 4.0 (Raymond and Rousset 1995). F_{ST} values were estimated, as in Weir and Cockerham (Weir and Cockerham 1984), among pairs of populations. The modified Garza-Williamson (G-W) index (the number of alleles divided by the allelic range plus one, was calculated for all the populations using ARLEQUIN. The G-W index is expected to be low in bottlenecked populations. The non-parametric Wilcoxon singed-rank test was used to compare the two bottlenecked populations Brisbane Ranges and Stony Rises to the non-bottlenecked Queensland (zoo) population.

Population structure was examined using the software STRUCTURE 2.3.2 (Pritchard et al., 2000). The software STRUCTURE is a model-based clustering method that takes a Bayesian approach to identify distinct genetic populations and assign individuals (probabilistically) to these populations. The software assumes a model where there are *K* populations (where *K* may be unknown), each of which is characterized by a set of allele frequencies at each locus. STRUCTURE attempts to assign individuals to "populations" on the basis of their genotypes, while simultaneously estimating population allele frequencies. We applied this method using 13 (*Phci15* was eliminated from analysis due to linkage disequilibrium) microsatellites markers in three koala populations. It assumed that within populations, the loci are at Hardy-Weinberg

equilibrium, and linkage equilibrium. Individuals in the samples are assigned to populations, or jointly to two or more population if their genotypes indicate that they are admixed.

Four models were used to examine the effects of various combinations of assumptions of individual genetic ancestry and genetic relatedness among populations: 1) admixture with correlated allele frequencies; 2) admixture with independent allele frequencies; 3) no admixture with correlated allele frequencies; and 4) no admixture with independent allele frequencies. Each model was run 10 times using values of K (possible number of populations) between 1 and 8 to give a large margin of error in our estimates of maximum number of populations, and to allow for possible genetic structuring within each site. Each run was performed with a burn-in of 50,000 iterations, followed by 500,000 Markov chain Monte Carlo iterations.

Population structure was estimated using 22 samples from Brisbane Ranges, 24 samples from Stony Rises and 27 koala samples from Queensland (zoo), using genotypes for 13 microsatellite markers in each population. STRUCTURE was also run with K = 1 through K = 3 for a dataset consisting of just the two southern populations: Brisbane Ranges and Stony Rises.

The value of *K* was estimated using two approaches: use of *ln P(D)* and the use of an *ad hoc* quantity ΔK. When K is approaching a true value, *ln P(D)* plateaus, and has high variance between runs (Pritchard et al., 2000). Delta K, is calculated based on the second order rate of change of the likelihood (ΔK) (Evanno et al., 2005). The ΔK shows a clear peak at the true value of K. The uppermost hierarchical level of population structure was examined using the *ad hoc* statistics delta K based on the rate of change in *ln P(D)* between successive K values (Evanno et al., 2005), implemented in Structure Harvester (Earl and Vonholdt 2012).

To further examine the genetic relationships among detected clusters, principal

coordinate analysis (PCoA) was conducted using two datasets using GenAlEx. The first dataset included 13 microsatellite markers, the two southern populations and the Queensland (zoo) population. The second data set included the same number of markers and the southern populations only. PCoA is a multivariate technique that allows one to find a plot the major patterns within a multivariate data set (e.g., multiple loci and multiple samples). In essence, PCoA is a process by which the major axes of variation are located within a multidimensional data set. For multidimensional data sets, each successive axis explains proportionately less of the total variation, such that when there are distinct groups, the first two or three axes will typically reveal the most of the separation among them (Peakall and Smouse 2012). The PCoA method uses a genetic distance matrix generated in GenAlEx to search for differences and similarities of the genetic data and plot the patterns to the principal coordinates.

Results

Out of the 14 markers, three were monomorphic (*Phci21*, *Phci23* and *Phci27*) in all koalas from Brisbane Ranges, while two (*Phci23* and *Phci27*) were monomorphic in Stony Rises. Nine markers were polymorphic in Brisbane Ranges and ten markers were polymorphic in Stony Rises. All fourteen loci were highly polymorphic in koalas from the Queensland (zoo) population (Table 4). Allelic diversity ranged from 4 (*Phci30*) to 13 (*Phci22*) alleles per locus with an average of 7.71 alleles per locus per population (Table 4). The Brisbane Ranges and Stony Rises koala population had a lower number of alleles at each locus compared to those from Queensland (zoo) koalas. The Brisbane Ranges population had 2.5 alleles per locus, and Stony Rises had 2.7 alleles per locus. The koalas from Queensland (zoo) had a broader allele size distribution for most loci and exhibited 5 private alleles per locus and 6.8 alleles per locus (Figure 5).

Among the 14 koala microsatellite markers there were 9 loci that had more than one missing allele within the size range of alleles detected: Locus *Phci2* had five missing alleles (144, 148, 150, 154, and 168); locus *Phci5* also had five missing alleles (157, 161, 165, 169, and 173); locus *Phci9* had two missing alleles (172 and 176); *Phci10* had four missing alleles (196, 214, 218, and 222); *Phci15* had nine missing alleles (208, 210, 212, 214, 216, 221, 225,229, and 233); *Phci18* had five missing alleles (159, 161, 163, 165, and 167); *Phci19* had two missing alleles (173 and 177); *Phci23* had three missing alleles (182, 188, and 190); *Phci24* had two missing alleles (183 and 185); lastly, *Phci27* had one missing allele (198) (Figure 5).

Allele size ranges in Brisbane Ranges and Stony Rises were the same for most loci. Brisbane Range and Stony Rises shared the same alleles except in two loci. In locus *Phci10*, allele 216 was found only in one individual in Brisbane Ranges and in locus *Phci18* allele 199 was found in one individual from Stony Rises. In addition, the Stony Rises population had four alleles that were shared with the Queensland (zoo) population and were not found in the koalas from Brisbane Ranges. The four alleles were: allele 180 in locus *Phci21*, allele 194 and 198 in locus *Phci10*, and allele 177 in locus *Phci18*.

Overall mean observed heterozygosity was Ho = 0.51 and ranged from a low Ho = 0.39 in the Brisbane Ranges population to a high of Ho = 0.72 in the Queensland (zoo) population (Table 4). Between pairs of loci, no significant linkage disequilibrium was detected after Bonferroni correction ($P < 0.0005$), except for one marker (*Phci15*).

Evidence of deviation from Hardy-Weinberg disequilibrium was detected in marker *Phci17* in the Brisbane Ranges and Stony Rises populations but not in the Queensland (zoo). All other loci were under Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.0005$).

Measures of genetic differentiation between populations suggested moderate amounts of genetic distance between the koala populations. Low genetic differentiation was observed between Brisbane Ranges and Stony Rises (F_{ST} = 0.02). High levels of genetic differentiation was observed between the Queensland (zoo) population and the two southern koala populations, Brisbane Ranges and Stony Rises (F_{ST} = 0.32 and F_{ST} = 0.29, respectively).

The average value of modified G-W index was 0.31 and 0.36 for Brisbane Ranges and Stony Rises, respectively and 0.76 for the Queensland (zoo) population (Figure 5). The modified G-W indices were significant after Bonferroni correction for multiple comparisons ($P < 0.025$).

The STRUCTURE results suggested the presence of 2 genetically distinct clusters corresponding to koalas at: 1) Queensland (zoo), and 2) Brisbane Ranges and Stony Rises (Figure 2). The *ad hoc ΔK* method implemented in STRUCTURE HARVESTER estimated a total of two clusters (K = 2) for all models. The *K* values assigned when using the maximum *ln P(D*) method differed and were higher for all methods than when *ΔK* method was implemented. The number of clusters assigned based on the *ln P(D)* method was K = 4 for models with correlated allele frequencies and K = 3 for models with independent allele frequencies (Evanno et al., 2005). Based on proportions of alleles assigned to particular populations, the STRUCTURE results suggest that individuals from the Queensland (zoo) koala populations had a different genetic composition from those of the other populations, regardless of model assumptions. No unique clusters were observed when STRUCUTRE analysis included only individuals from the two southern populations (data not shown).

The PCoA analysis implemented using the software GenAlEx showed distinctiveness between northern and southern koalas populations. The first principal component separates the

Brisbane Ranges and Stony Rises koalas from the Queensland (zoo) koalas (Figure 8). There is little distinctiveness between Brisbane Ranges and Stony Rises group as indicated by their proximity to each other in the PCoA (Figure 8). The Queensland (zoo) group is separated from the southern koalas, which indicates that the two groups are genetically distinct. The second PCoA (Figure 9) revealed no discrete separation between Brisbane Ranges and Stony Rises koala samples, indicating that these two populations are not highly distinguishable.

Discussion

Here we studied koalas from Brisbane Ranges and Stony Rises, two southern Australia koala populations that have experienced population decline after being hunted in the early 1900s. These two southern populations were also recipients of a translocation program in southern Australia and were repopulated with koalas from a highly inbred koala population on French Island, an island off the southern coast of Victoria. French Island koalas were the source population of almost all populations reintroduced in Victoria and South Australia (Martin et al., 1999). Large numbers of koalas (>1200) were translocated to Stony Rises and more than 260 to Brisbane Ranges (Taylor et al., 1997).

The low levels of genetic differentiation, heterozygosity and allele frequencies found by this study are probably due to the combined effects of small population size and founding events. In populations with a small number of founders, genetic drift acts quickly, increasing the chance for rare alleles to be lost, which can further decrease the gene pool, a process referred to as the founder effect. In the event that the population size is reduced to a really small number a bottleneck effect occurs, and the more generations a bottleneck lasts the more alleles the population losses (Hedrick 2005).

The severe genetic consequences of population bottlenecks, founder events and translocations on koalas was demonstrated by the low levels of both allelic diversity and heterozygosity present in two southern koala populations. Our study demonstrated that microsatellite variability was low in samples collected from Brisbane Ranges (Ho = 0.39) and Stony Rises (Ho = 0.40) compared to the Queensland (zoo) population (Ho = 0.72). Houlden et al., (1996b), compared populations from southeastern Australia with koala populations from northeastern Australia, and also revealed low levels of heterozygosity in the southern populations (He = 0.436). In this study, the koalas from Queensland (zoo) also showed high levels of microsatellite variation (Ho= 0.739). Furthermore, the limited allele size range found in the koala populations from southern Australia suggests that rare alleles may have been lost following the severe bottleneck. With only five alleles per locus (Table 4), the two southern koala populations show low levels of genetic diversity consistent with their history of population decline after severe hunting and repopulation with inbred koalas from the French Island population. The modified Garza-Williamson values were low, ranging from 0.11 to 0.62 in the Brisbane Ranges and Stony Rises koalas, suggesting past population size reductions (Figure 6).

Observable differences in allelic distributions were apparent in our studied koala populations. The Queensland (zoo) koalas had broader size range and had higher allelic diversity than the Brisbane Ranges and Stony Rises populations. Unlike the southern populations, which showed one or two predominant alleles at each locus, Queensland (zoo) population had more alleles but in lower frequencies. The Queensland (zoo) allele size distribution is disjunct or non-continuous in the majority of the loci, which indicates that there are missing intermediate allele sizes. As for the southern koala populations, there are large gaps that are separated by several missing alleles. The disjunct allele pattern in southern koalas could be explained by limited allele

sampling caused by a recent founder effect in which a small number of alleles is observed. This disjunct pattern contrasts the continuous distribution of microsatellite loci as predicted by the stepwise mutation model (SMM) for microsatellite, which suggests that microsatellites increase or decrease by one unit or repeat per mutation event. The random increase or decrease in allele frequencies through genetic drift would also cause allele distributions to develop gaps and irregular patterns.

The results from STRUCTURE indicate that there is genetic differentiation among the three koala populations (Figure 7). By examining microsatellite allele frequencies, STRUCTURE identified two separate clusters (K =2), which separated the Queensland (zoo) population from the southern koala populations. The koalas from Queensland (zoo) were distinctive from the Brisbane Ranges and Stony Rises koalas, exhibiting high genetic differentiation (Table 5), distinct clustering (Figure 7 and 8) and unique allele frequency patterns (Figure5).

The clustering of genetically different groups was further supported by principal coordinate analysis (Figure 8 and 9). PCoA found and plotted two major groups based on the patterns found within a distance matrix generated by pairwise individual-by-individual comparisons. The first group was defined by koala genotypes from Brisbane Ranges and Stony Rises which were similar to each other and therefore closer. The second group consisted of samples from Queensland (zoo), which were completely separated from the southern samples. The major groups found by PCoA mirror the clustering pattern revealed by the STRUCTURE analysis.

The lack of genetic differentiation between Brisbane Ranges and Stony Rises populations shown by the cluster analysis (Figure 7) and their interspersed pattern (Figure 9) are due to their similarities in allele diversity (Table 4) and allele frequencies (Figure 5). A similar conclusion was derived by a study that used minisatellites to evaluate variation in koalas from Phillip Island, Brisbane Ranges and south Gippsland. The minisatellites results showed almost no differentiation across the populations (Taylor et al., 1991).

Comparison of mtDNA haplotypes and microsatellites DNA patterns

In our previous analysis using mtDNA control region (see Chapter 2), we compared haplotypes of seven southern koala populations, including Brisbane Ranges and Stony Rises, to seven population from northern Australia. We found that there was one common haplotype (KCR1) shared among most of the southern populations. Haplotype KCR1 was the only mtDNA haplotype found in koalas from Brisbane Ranges, Stony Rises, French Island, Phillip Island, and Kangaroo Island. This is not surprising, since these populations were repopulated with koalas from the same inbred source population (French Island). Our mtDNA analysis show that the Brisbane Ranges and Stony Rises populations, which are geographically distinct, are not demographically independent due to the large numbers of koalas that were translocated to these locations from the same highly inbred source population in French Island. While our koalas from southern Australia had limited haplotype diversity, eight haplotypes were detected in our koala samples from Queensland (zoo).

The low mitochondrial DNA diversity found in koalas from Victoria is in accordance with our microsatellite results. Analyses of 14 microsatellites showed that allelic diversity was low in the koala populations from Brisbane Ranges and Stony Rises. The number of alleles ranged from 1 to 5 alleles per locus in Brisbane Ranges and Stony Rises populations. One

microsatellite was found to be in significant deviation from HWE in both populations. This may be caused by a number of factors, including nonrandom mating; positive or negative assortative mating; inbreeding; small population size, selection, subdivided populations; gene flow or genetic drift (Lee et al., 2010).

Our results have shown that koalas from Brisbane Ranges and Stony Rises share the same mtDNA haplotype with koalas from French Island. We also found low allele diversity in the two southern populations. The limited numbers of microsatellite alleles found in the two southern populations were also in high frequencies, indicating that the alleles may have been increased by random genetic drift caused by a small population size. The effects of genetic drift may cause large and random changes in allele frequencies in small populations.

Conclusions

Past population declines leading to loss of genetic diversity through founder effects and bottlenecks have affected the genetic diversity of the koalas in Brisbane Ranges and Stony Rises. Our microsatellite DNA analysis showed that Brisbane Ranges and Stony Rises had limited allele diversity, compared to higher allele diversity in koalas from Queensland (zoo). Analyses for the detection of bottleneck showed evidence for past population size reduction and loss of alleles. Furthermore, we detected high genetic differentiation between Queensland (zoo) koalas and Victorian koalas. In accordance with the low genetic variability detected with neutral markers, mtDNA analysis revealed low haplotype diversity in southern koalas compared to Queensland (zoo) koalas. Sequencing 648 bp of the mitochondrial control region revealed that our koala samples from the Brisbane Ranges and Stony Rises carry only one haplotype, while the koalas from Queensland (zoo) carry eight mtDNA haplotypes. The findings of this study have increased our understanding of koala population structure and genetic diversity, and may have

implications for wild koala management strategies in Victoria, Australia and for the management of koalas in zoos in the US.

Tables and Figures

Table 4. Fourteen microsatellite markers developed in the koala (*Phascolarctos cinereus*).

	Brisbane Ranges					Stony Rises					Queensland (zoo)				
Locus	*N*	Size (bp)	A	H_E	H_O	*N*	Size (bp)	A	H_E	H_O	*N*	Size (bp)	A	H_E	H_O
Phci2[a]	22	158-170	2	0.30	0.27	24	158-170	2	0.25	0.21	27	142-172	10	0.84	0.78
Phci5[a]	22	163-171	3	0.61	0.77	24	163-171	3	0.60	0.63	27	155-175	6	0.82	0.89
Phci9[a]	22	170-174	2	0.44	0.45	24	170-174	2	0.40	0.30	27	168-182	5	0.66	0.59
Phci10[a]	22	204-220	4	0.59	0.55	24	194-220	5	0.65	0.50	27	194-224	9	0.86	0.89
Phci15[a]	21	219-223	2	0.46	0.48	21	219-223	2	0.50	0.61	27	206-235	6	0.73	0.70
Phci17[*] [a]	22	153-167	5	0.73	0.68	24	153-167	5	0.75	0.83	27	155-167	7	0.82	0.85
Phci18[a]	22	171-175	3	0.21	0.23	24	171-177	4	0.50	0.50	27	157-181	7	0.67	0.70
Phci19[a]	21	179-183	3	0.52	0.48	23	179-183	3	0.65	0.52	27	167-175	4	0.38	0.41
Phci21[b]	22	178	1	0	0	24	178-180	2	0.04	0.04	27	178-192	7	0.84	0.89
Phci22[a]	22	185-196	4	0.73	0.64	24	185-196	4	0.67	0.58	27	183-208	11	0.78	0.78
Phci23[b]	19	184	1	0	0	21	184	1	0	0	27	178-194	6	0.74	0.70
Phci24[b]	20	179-193	2	0.41	0.45	24	179-193	2	0.50	0.41	26	177-195	7	0.78	0.58
Phci27[a]	22	196	1	0	0	24	196	1	0	0	27	192-208	8	0.84	0.93
Phci30[b]	20	195-197	2	0.47	0.5	22	195-199	3	0.52	0.50	26	195-201	3	0.49	0.65

N is the number of individuals successfully amplified, H_O is observed heterozygosity, H_E is expected heterozygosity, bp (base pairs)
A is the number of alleles for each loci in each population
*Significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.0005$)
[a] Loci from Ruiz-Rodriguez *et al.*, 2014
[b] Loci characterized for the first time in this study

Table 5. Pairwise F_{ST} for all pairs of populations. F_{ST} shows significant differentiation between koalas from Brisbane Ranges (BR), Stony Rises (SR) and Queensland Zoo abbreviated as QLD (zoo). Population pairwise F_{ST} is shown below diagonal and *P* values are above diagonal. Comparison of QLD (zoo) with koalas from BR and SR show that koalas from QLD (zoo) are genetically distinct. However, koalas from BR and SR are genetically similar as indicated by a low $F_{ST.}$

	BR	SR	QLD (zoo)
BR	–	0.009	<0.001
SR	0.03	–	<0.001
QLD (zoo)	0.32	0.29	–
	BR	SR	QLD (zoo)

Figure 5. Allele frequencies for 14 koala microsatellite markers among three populations: Brisbane Ranges (blue), Stony Rises (orange) and Queensland (zoo) (gray). Each bar graph shows the frequency of alleles in each locus in three koala populations. The vertical axis shows the allele frequencies within each population. The horizontal axis shows the alleles found in each locus. Missing alleles are indicated by black vertical lines between the allele sizes. Loci *Phci23* and *Phci27* were monomorphic in Brisbane Ranges and Stony Rises and *Phci21* was monomorphic in Brisbane Ranges. The Brisbane Ranges and Stony Rises koalas carry a relatively small number of alleles, indicating loss of alleles through a founder effect and high frequencies caused by genetic drift. The Queensland (zoo) koalas show a higher number of alleles with lower frequencies.

Figure 5 (cont.)

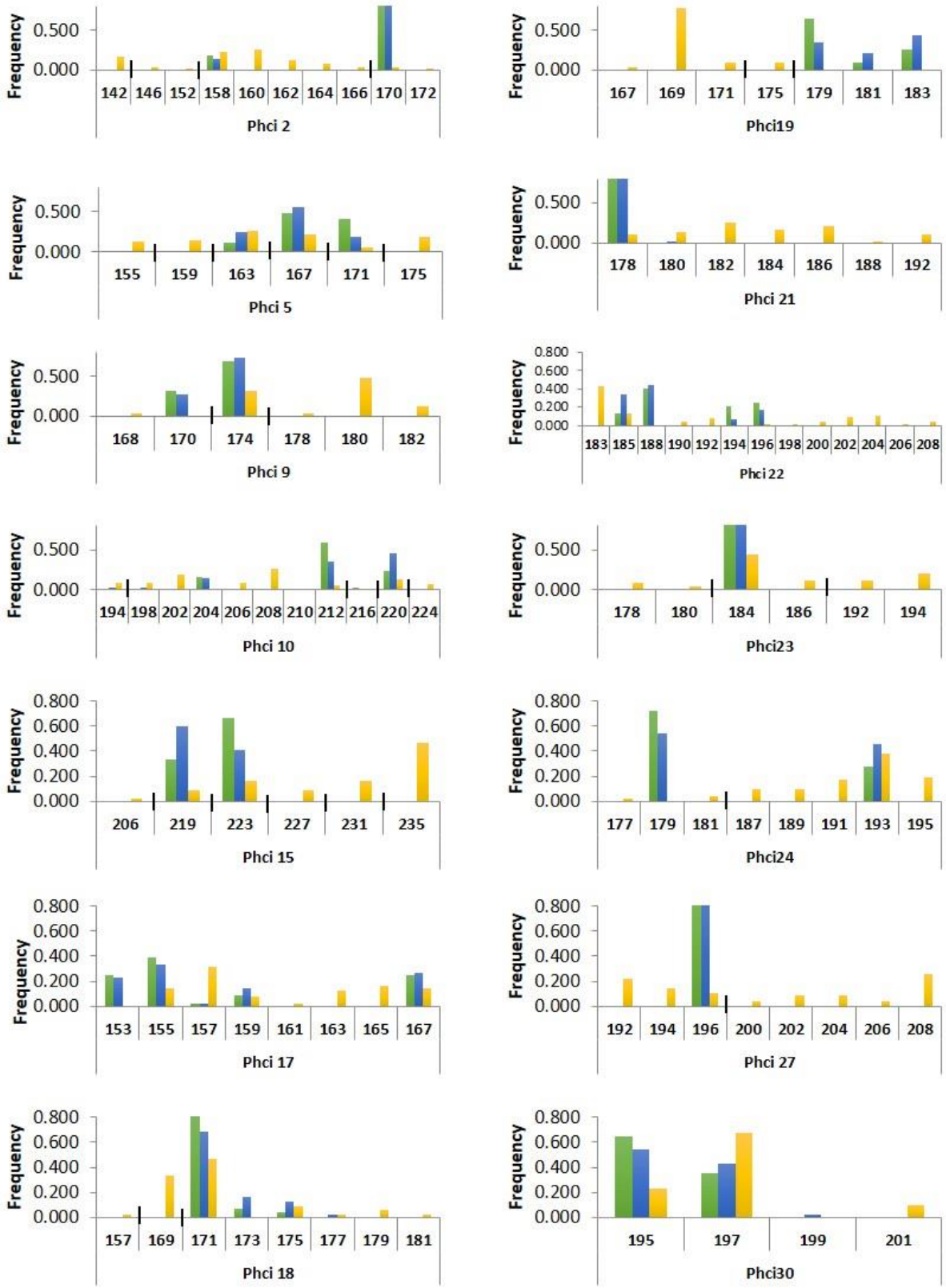


Figure 6. Modified Garza-Williamson (G-W) index for 13 microsatellite loci for three koala populations: Brisbane Ranges (BR), Stony Rises (SR) and Queensland (zoo). Each bar represents a locus. The vertical axis shows the modified G-W index and the horizontal axis represents one locus. The G-W statistic is given as G-W = k / R +1 where k is the number of alleles at a given locus in a population sample, and R is the allele size range. Originally, the denominator was defined as just R in Garza and Williamson (2001), but this could lead to a division by zero if a sample is monomorphic. The modified Garza-Williamson index was introduced in Excoffier et al., (2005). The G-W statistic was shown to be sensitive to population bottleneck, because the number of alleles is usually more reduced than the allele range by a recent reduction in population size. The average value of modified G-W index was 0.31 and 0.36 for Brisbane Ranges and Stony Rises, respectively and 0.76 for the zoo population of Queensland koalas. The G-W value given to each locus in the BR and SR population was compared to the G-W value given to each locus in the QLD (zoo) population. Wilcoxon test for significance showed that G-W values were significant after Bonferroni correction ($P < 0.025$).

Figure 6 (cont.)

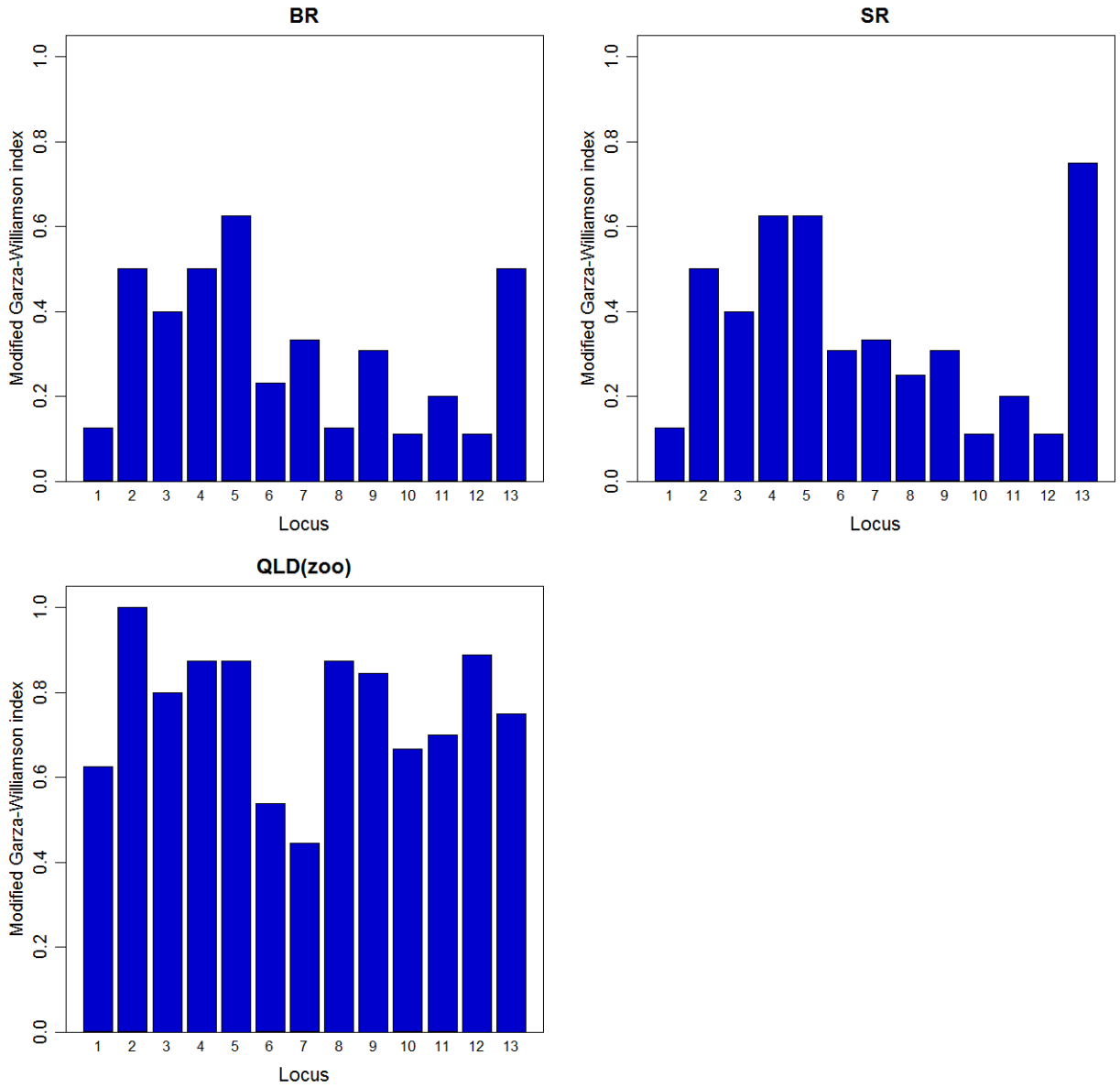


Figure 7. Genetic partitioning of koala populations using the program STRUCTURE. The two partitions largely correspond to two southern koala populations (green) and the Queensland (zoo) population (red). Each individual is represented by a column and the probability of that individual belonging to each of the K populations is indicated by colored segments. Based on microsatellite allele frequencies, the two southern populations were assigned to the cluster colored in green. In the southern koala partition sample EX1279 (indicated by arrow), 80% corresponds to one population and 20% to another, although this might result from allelic drift and not from any admixture by Queensland koalas.

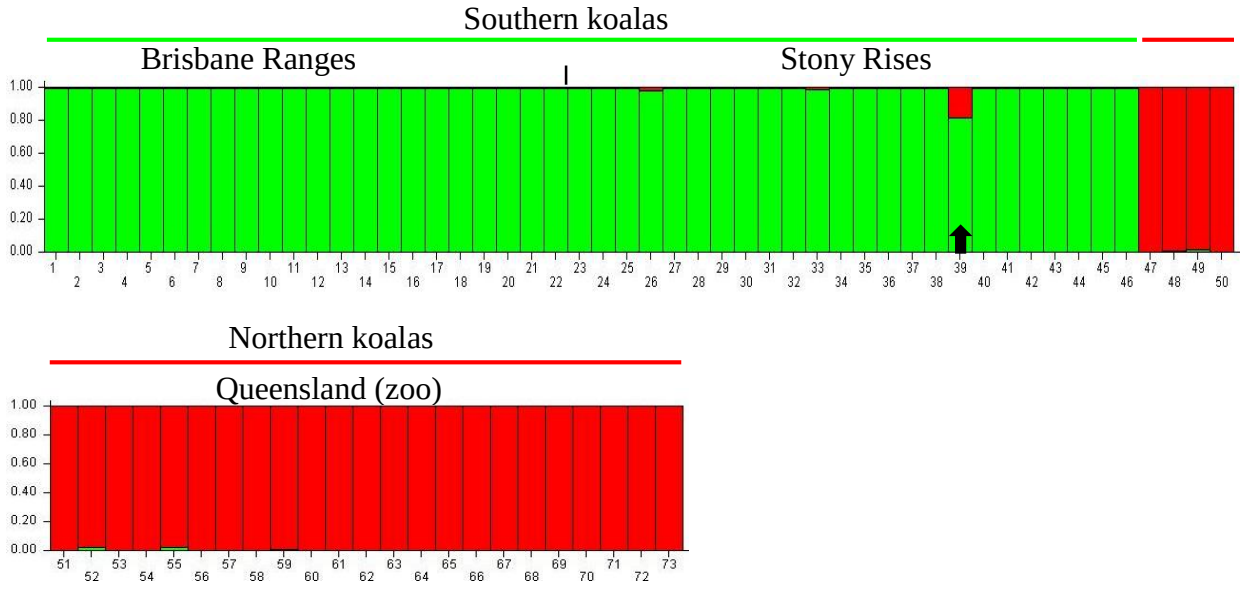


Figure 8. Principal coordinate analysis (PCoA) of three koala populations. The genotype of each individual is represented by a circle on the graph. Different color circles were used for each locality: Brisbane Ranges (green), Stony Rises (blue) and Queensland (zoo) yellow. Variation explained along axes 1, 2, and 3 was 31.39 %, 6.34% and 6.09%. Axis 3 is not shown and was not informative across populations. The koalas from Brisbane Ranges (BR) and Stony Rises (SR) are separated from the Queensland (zoo) koalas along axis 1. Koalas from Brisbane Ranges and Stony Rises are similar to each other as indicated by the blue and green circles, while the Queensland (zoo) koalas (yellow circles) are different from the BR and SR koalas. The PCoA shown here indicates that SR and BR koala populations are genetically distinct from QLD (zoo).

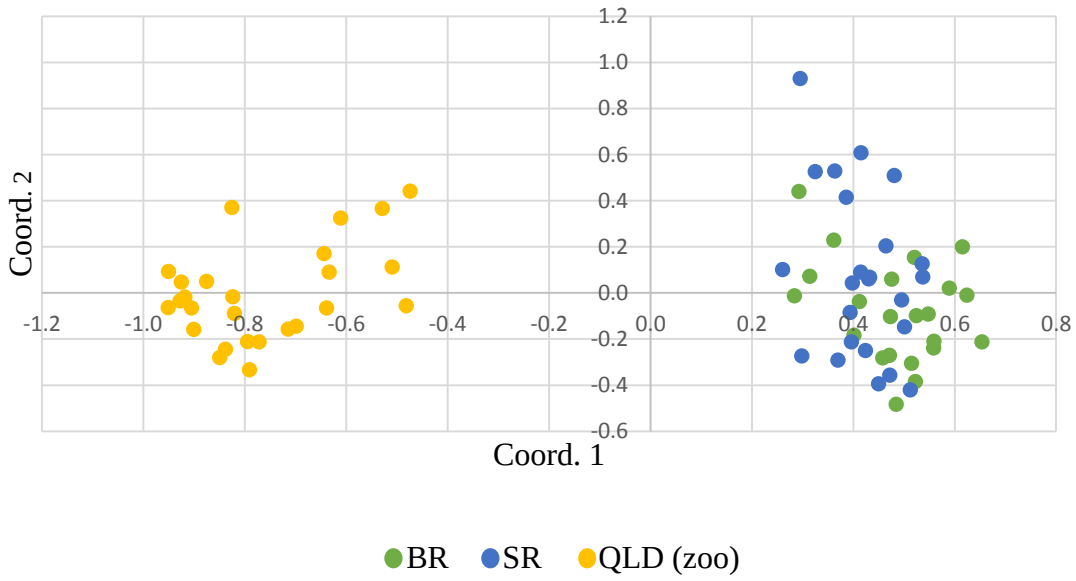
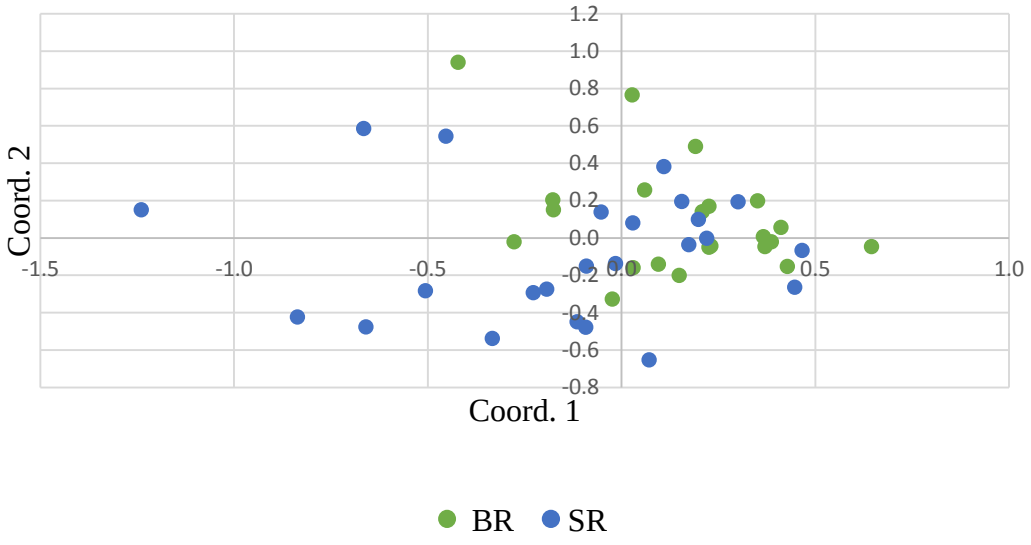


Figure 9. Principal coordinate analysis (PCoA) plot for Brisbane Ranges (BR) and Stony Rises (SR) koala populations. The genotype for each individual is represented by a circle on the graph: BR (green) and SR (blue). The first principal coordinate accounts for 14.49% of the total variation, while the second principal coordinate account for 11.31% of the total variation. The two populations are not segregated into discrete groups, but rather overlap with each other, indicating that they are genetically similar.



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